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Influence of various antioxidants added to TCM-199 on post-thaw bovine sperm parameters, DNA integrity and fertilizing ability

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ABSTRACT

Supplementation of the semen extender with antioxidants did not produce any significant effect on CASA and progressive motilities and sperm motility characteristics, in comparison to the control group ($P > 0.05$).

For sperm acrosome and total abnormalities, TCM-199 supplemented with cysteine (2.60 ± 0.24% and 4.80 ± 0.20%), glutamine (2.80 ± 0.20% and 6.40 ± 0.40%), carnitine (2.60 ± 0.24% and 6.00 ± 0.63%) and methionine (3.40 ± 0.51% and 9.20 ± 0.86%) at doses of 2 mM provided a better protective effect, compared to that of the controls (8.00 ± 0.44 and 15.60 ± 1.895). As regards sperm membrane integrity, supplementation with 2 mM of glutamine and methionine (56.00 ± 1.70% and 62.40 ± 1.78%, respectively) resulted in higher rates, when compared to the control group (41.40 ± 4.74%). According to the results of the COMET assay, only the use of TCM-199 supplemented with 2 mM of cysteine reduced DNA damage and resulted in percentages of sperm with damaged DNA (2.17 ± 0.18%) lower than those of the control group (3.16 ± 0.32%) ($P < 0.001$). For pregnancy rates, there were no significant differences among the extender groups ($P > 0.05$).

Introduction

Damage induced by cryopreservation has been attributed to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid–protein reorganizations within the cell membrane [11,55]. Oxidative damage caused by reactive oxygen species (ROS) leads to impaired cell functions, which result in the loss of sperm motility, morphological integrity and fertilizing capability, and the induction of sperm apoptosis [3,26]. It is well known that mammalian spermatozoa contain high concentrations of polyunsaturated fatty acids, and therefore, are highly vulnerable to oxidative stress, which is responsible for the generation of ROS [3,22]. The cell membrane is thought to be a primary target of freezing or cold shock damage in cells [2,4]. The presence of high concentrations of long-chain polyunsaturated fatty acids (LC-PUFA) within the lipid structure of cell membranes requires efficient antioxidant systems to defend against peroxidative damage and associated sperm dysfunction [3,5]. Spermatozoa and the seminal plasma possess an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) to prevent oxidative damage. However, the antioxidant capacity of sperm cells is limited due to only a small cytoplasmic component containing antioxidants to scavenge oxidants [48]. Thus, mammalian spermatozoa may fail in preventing lipid peroxidation (LPO) during the freeze–thawing process [10].

Additives with antioxidative properties have been reported to reduce the impact of ROS-induced and cold shock damages [6,23], and thus, to improve ram [1,14], goat [16], boar [25], canine [35] and human [5] sperm quality. Mammalian cells can utilize only thiol compounds, such as cysteine and GSH, for intracellular GSH biosynthesis both in vitro and in vivo, as these compounds can easily penetrate the cell membrane. GSH protects membrane lipids and proteins by its direct radical-scavenging properties [34,44]. Cysteine has been shown to improve the post-thaw motility and morphology of bull [12,45], ram [53] and goat [15] sperm, and to maintain the viability, chromatin structure and membrane integrity of boar sperm during liquid storage at 5 °C [50].

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Additionally, it has been demonstrated to improve porcine oocyte maturation and fertilization in vitro [31].

Amino acids (AAs) protect the membrane structure of sperm cells during the freeze–thawing process. Some plants and animals accumulate amino acids in response to exposure to freezing temperatures. Amongst amino acids used for the supplementation of freezing extenders, glutamine has been reported to be used against damage caused by freeze–thawing in the semen of several animal species, including the Angora goat [16,19], ram [20], stallion [32] and bull [8].

Carnitine, a vitamin-like compound, is biosynthesized from two essential amino acids, namely, lysine and methionine, in the liver, kidneys and brain. L-carnitine is found at high concentrations in the mammalian epididymis and spermatozoa. It is involved in the generation of metabolic energy by facilitating the transportation of fatty acids into the mitochondria. Epididymal cells and spermatozoa derive energy from carnitine found in the epididymal fluid [24,33]. The strong correlation between seminal carnitine and semen quality has been demonstrated in various models (ram, human, stallion and rat) [13,43,49].

Methionine acts as a precursor amino acid of glutathione for protecting cells from oxidative damage, and plays a vital role in detoxification [40,41]. In addition, the thiol group of methionine has been shown to chelate lead and remove it from tissues [38].

The composition of the semen extender is highly important for semen cryopreservation. To our knowledge, the role of antioxidants added to TCM-199 against the cryodamage of bovine sperm has not been investigated before. The novelty of this study was to determine the effects of various antioxidants added to TCM-199, prior to cryopreservation, on in vitro sperm parameters (motility, acrosome and total abnormalities, membrane and DNA integrities) and in vivo fertilizing ability for frozen-thawed bovine sperm.

Materials and methods

Chemicals

The antioxidants (cysteine, carnitine, glutamine and methionine) and other chemicals used in this study were obtained from Sigma–Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Animals and semen collection

Ejaculates from three Holstein bulls (3 and 4 years of age) were used in the study. The bulls, belonging to the Lalahan Livestock Central Research Institute (Ankara, Turkey), were maintained under uniform feeding and housing conditions. A total number of 36 ejaculates (12 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. Only ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/ml were used. Immediately after collection, the ejaculates were immersed in a warm water bath at 34 °C until their assessment in the laboratory.

Semen processing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). Sperm motility was estimated using phase-contrast microscopy (200×). The TCM-199 medium (egg yolk 15% (v/v), glycerol 6% (v/v), pH 6.8) was used as the base extender (freezing extender). Each ejaculate was split into five equal parts and diluted to a final concentration of 60×10^6/ml spermatozoa with the TCM-199 base extender containing cysteine (2 mM), carnitine (2 mM), methionine (2 mM) and glutamine (2 mM) and no additive (control). Diluted semen samples were loaded into 0.25-ml French straws and cooled down to 4 °C in 2 h, frozen at a programmed rate of −3 °C/min from +4 to −10 °C; −40 °C/min from −10 to −100 °C; and −20 °C/min from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. After at least 24 h, the frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

Semen evaluation

Evaluation of sperm parameters

Analysis of subjective and CASA motilities. A computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to analyze sperm motility and motility characteristics. CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size – 10 pixels; default cell intensity – 80. Thawed semen was diluted (5 μL semen +95 μL extender) in a TCM-199 extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-μL sample of diluted semen was put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland) and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility, VAP (average path velocity, μm/s), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of lateral head displacement, μm), and LIN (linearity index, %). For each evaluation, 10 microscopic fields, including at least 300 cells, were analyzed.

Assessment of sperm abnormalities

For the assessment of sperm abnormalities, at least three drops of each sample were added into Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water) [46]. One drop of this mixture was put onto a slide and covered with a cover slip. The percentages of sperm acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under a phase-contrast microscope (magnification 1000×, oil immersion).

Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution (9 g fructose +4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Two hundred sperm were evaluated (magnification 1000×) with bright-field microscopy. Sperm with swollen or coiled tails were recorded [21,42].

Fertility trials

The fertilizing ability of spermatozoa was calculated based on the non-return rates at 59 days. A total of 230 cows were artificially inseminated with frozen antioxidant-supplemented samples and control samples, each comprising the semen of one bull. The effective non-return rates (NNR) were determined at 59 days post-insemination by palpation per rectum.

Table 1
Mean (±SE) sperm motilities in semen supplemented with different antioxidants of Holstein bovine semen following freeze–thawing.

Groups	Progressive motility (%)	CASA motility (%)
Cysteine 2 mM	9.43 ± 1.63	39.25 ± 5.63
Glutamine 2 mM	7.71 ± 1.43	26.80 ± 1.16
Carnitine 2 mM	9.40 ± 1.63	28.80 ± 6.22
Methionine 2 mM	6.50 ± 1.61	23.25 ± 3.28
Control	10.43 ± 2.09	27.67 ± 2.49
P	–	–

–: The same column shows no significant differences among proportions ($P > 0.05$).

Assessment of sperm DNA damage

Diluted semen samples were centrifuged at 300*g* for 10 min at 4 °C. The seminal plasma was discarded and the remaining sperm cells were washed with (Ca2+ and Mg2+ free) PBS to yield a concentration of 1×10^5 spermatozoa/cm^3 [9].

Sperm DNA damage was investigated using the single cell gel electrophoresis (COMET) assay, which is generally performed at high alkaline conditions. Firstly, each microscope slide was precoated with a layer of 1% normal melting point agarose in PBS and dried at room temperature. Next, 100 μl of 0.7% low melting point agarose at 37 °C was mixed with 10 μl of the cell suspension and poured on top of the first layer. The slides were allowed to solidify for 5 min at 4 °C in a moist box. The cover slips were removed and the slides were immersed in a freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na2–EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO and 40 mM dithiothreitol (pH 10) for 1 h at 4 °C. Subsequently, the slides were incubated overnight at 37 °C in 100 μg/ml proteinase K (Sigma) and placed into lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with a fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 8 °C at 12 V and was adjusted to 250 mA. Next, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove the alkali and detergents. After neutralization, the slides were stained with 50 μl of 2 μl/ml ethidium bromide and covered with a cover slip. All steps were performed under dim light to prevent further DNA damage [29,47].

The images of 50 randomly chosen nuclei were analyzed by CASP. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, BX51, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered to be damaged.

Statistical analysis

Results were expressed as mean ± SEM. Sperm motility, motility characteristics, abnormalities and biochemical data were analyzed by analysis of variance, followed by Tukey's post hoc test to determine significant differences between the groups. The groups were compared for DNA damage, using analysis of variance and Tamhane's T2 multiple range test as a post hoc test. The groups were also compared for pregnancy rate, using Pearson's chi-square test. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed using the SPSS 11.5 package program.

Results

Supplementation of the semen extender with antioxidants did not produce any significant effect on CASA and progressive motilities and sperm motility characteristics, in comparison to the control group (Tables 1 and 2, $P > 0.05$).

The supplementation of TCM-199 with cysteine (2.60 ± 0.24% and 4.80 ± 0.20%), glutamine (2.80 ± 0.20% and 6.40 ± 0.40%), carnitine (2.60 ± 0.24% and 6.00 ± 0.63%) and methionine (3.40 ± 0.51% and 9.20 ± 0.86%) at doses of 2 mM provided a better protective effect against sperm acrosome and total abnormalities, compared to the controls (8.00 ± 0.44 and 15.60 ± 1.895). As regards sperm membrane integrity, supplementation with 2 mM of glutamine and methionine (56.00 ± 1.70% and 62.40 ± 1.78%, respectively) resulted in higher integrity rates, when compared to those of the control group (41.40 ± 4.74%). According to the results of the COMET assay, only the use of TCM-199 supplemented with 2 mM of cysteine reduced DNA damage and resulted in a rate of sperm with damaged DNA (2.17 ± 0.18%) lower than that of the control group (3.16 ± 0.32%) (Table 3, $P < 0.001$). For pregnancy rates, there were no significant differences between the extender groups (Table 4, $P > 0.05$).

Table 2
Mean (±SE) sperm motion characteristics in semen supplemented with different antioxidants of Holstein bovine semen following freeze–thawing.

Groups	VAP (mm/s)	VSL (mm/s)	VCL (mm/s)	ALH (mm)	LIN (%)
Cysteine 2 mM	100.47 ± 5.81	90.30 ± 5.07	153.40 ± 11.66	5.21 ± 0.45	63.14 ± 2.12
Glutamine 2 mM	90.39 ± 3.82	83.74 ± 3.90	131.93 ± 7.90	4.56 ± 0.42	70.43 ± 4.44
Carnitine 2 mM	97.16 ± 3.08	89.68 ± 2.58	135.24 ± 7.69	4.16 ± 0.31	68.80 ± 1.93
Methionine 2 mM	97.40 ± 4.18	89.72 ± 3.69	145.30 ± 8.16	5.02 ± 0.35	63.33 ± 1.50
Control	100.83 ± 4.62	93.09 ± 4.89	144.53 ± 8.36	4.50 ± 0.26	65.71 ± 1.30
P	–	–	–	–	–

–: The same column shows no significant differences among proportions ($P > 0.05$).

Table 3
Mean (±SE) sperm abnormalities. Membrane integrity and DNA damage in semen supplemented with different antioxidants of Holstein bovine semen following freeze–thawing.

Groups	Acrosomal abnormality (%)	Total abnormality (%)	Membrane integrity (%)	Damaged DNA (%)
Cysteine 2 mM	2.60 ± 0.24^{b}	4.80 ± 0.20^{c}	49.40 ± 3.06^{bc}	2.17 ± 0.18^{b}
Glutamine 2 mM	2.80 ± 0.20^{b}	6.40 ± 0.40^{bc}	56.00 ± 1.70^{ab}	3.49 ± 0.22^{a}
Carnitine 2 mM	2.60 ± 0.24^{b}	6.00 ± 0.63^{c}	42.60 ± 2.94^{c}	3.29 ± 0.30^{ac}
Methionine 2 mM	3.40 ± 0.51^{b}	9.20 ± 0.86^{b}	62.40 ± 1.78^{a}	2.38 ± 0.22^{bc}
Control	8.00 ± 0.44^{a}	15.60 ± 1.89^{a}	41.40 ± 4.74^{c}	3.16 ± 0.32^{ac}
P	**	**	**	**

$^{a–c}$ Different superscripts within the same column demonstrate significant differences ($^{**}P < 0.001$).

Table 4
Fertility results based on pregnancy rates after rectovaginal insemination with frozen–thawed bovine semen.

Groups	Pregnancy rate (%)
Cysteine 2 mM	45.2(19/42)
Glutamine 2 mM	50(24/48)
Carnitine 2 mM	49.6(23/49)
Methionine 2 mM	55.6(25/45
Control	47.8(22/46)
P	–

–: The same column shows no significant differences among proportions ($P > 0.05$).

Discussion

Sperm motility per se is crucial in facilitating the passage of sperm through the cervix and uterotubal junction. It is also important for the actual penetration of the cumulus cells and zona pellucida of the ovum [27]. For the antioxidants used in the present study, the findings obtained were in agreement with the results of a study performed by Bucak et al. [17] in cattle, which reported no significant improvement in motility characteristics, and CASA and progressive motilities of bovine sperm.

Motility analysis did not provide enough data for the evaluation of sperm quality after freeze–thawing. Sperm morphology has major importance for fertilizing capacity and the assessment of membrane functions. Highly motile spermatozoa may have damaged structure and function. On the other hand, non-motile cells may have an intact plasmalemma and acrosome and morphology integrity [53,54]. The acrosome contains enzymes required for the penetration of the spermatozoon through the ovum layers and zona pellucida during fertilization [27]. In the present study, total and acrosome abnormalities were observed to have decreased in the presence of antioxidants in the TCM extender. These results are in agreement with those reported by some researchers, suggesting that antioxidants improve the sperm total abnormalities for bovine [17] and the sperm acrosome and total abnormalities for goat [18] against cryodamage. Antioxidants protects membrane lipids and proteins by its direct radical-scavenging properties [34] and plays a vital role in detoxification [40], improving the sperm quality.

The sperm plasma membrane is rich in polyunsatured fatty acids, and is therefore, susceptible to peroxidative damage with consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility [6,7,28]. Long-term sperm storage leads to membrane deterioration due to membrane phase transitions occurring in the regions of the highly regionalized sperm plasma membrane. This study was undertaken to ascertain which agents and antioxidants, would provide the most effective protection against membrane damage during the freeze–thawing process. The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss of permeability under the stress of swelling driven by hypo-osmotic treatment. Thus, it provided a form of a membrane stress-test, which is particularly useful in testing the membrane-stabilizing action of antioxidants. The results presented clearly show that glutamine and methionine provided the strongest protective effect against cryodamage.

The COMET assay is a widely applied technique for measuring and analyzing DNA breakage in individual cells [37]. It has also been proven as a valid technique to evaluate whether antioxidants are able to maintain genetic material integrity in biological studies [30,36,51]. In addition to this, some authors suggest that sperm DNA integrity is a more objective marker of sperm function as opposed to sperm parameters such as motility [39,52]. In this study, the antioxidants carnitine and methionine could not maintain DNA integrity, compared to the control group. These results were contrary to those reported in a previous research on bovine sperm [17], in which the maintenance of DNA integrity was recorded.

With respect to the fertility results based on pregnancy rates, antioxidants do not seem to be an influential factor on the fertilizing ability of sperm following the freeze–thawing process. This result complies with previous studies reporting no improvement in fertility with the supplementation of the freezing extender [17,45,51].

In conclusion, it seems that, antioxidants improved post-thaw sperm acrosome damage and abnormalities. Furthermore, cysteine supplementation provided a better DNA integrity compared to the controls, following freeze–thawing. In view of the data presented, further insight is needed into the effects of different antioxidant doses for obtaining high fertility.

References

[1] E.G. Aisen, V.H. Medina, A. Venturino, Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations, Theriogenology 57 (2002) 1801–1808.
[2] R.J. Aitken, J.S. Clarkson, S. Fishel, Generation of reactive oxygen species, lipid peroxidation, and human sperm function, Biol. Reprod. 40 (1989) 183–197.
[3] R.J. Aitken, H. Fisher, Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk, BioEssays 16 (1994) 259–267.
[4] J.G. Alvarez, B.T. Storey, Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa, Mol. Reprod. Dev. 42 (1995) 334–346.
[5] J.G. Alvarez, B.T. Storey, Role of glutathione peroxidase in protecting mammalian antioxidant enzymes: their source, function in the female, and ability to preserve sperm DNA integrity in the golden hamster, J. Androl. 24 (1989) 704–711.
[6] J.G. Alvarez, B.T. Storey, Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility, Biol. Reprod. 29 (1983) 548–555.
[7] J.G. Alvarez, J.C. Touchstone, L. Blasco, B.T. Storey, Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa: superoxide dismutase as a major enzyme protectant against oxygen toxicity, J. Androl. 23 (1987) 338–348.
[8] L. Amirat-Briand, D. Bencharif, O. Vera-Munoz, H. Bel Hadj Ali, S. Destrumelle, S. Desherces, E. Schmidt, M. Anton, D. Tainturier, Effect of glutamine on post-thaw motility of bull spermatozoa after association with LDL (low density lipoproteins) extender: preliminary results, Theriogenology 71 (2009) 1209–1214.
[9] M. Arabi, Bull spermatozoa under mercury stress, Reprod. Dom. Anim. 40 (2005) 454–459.
[10] J.E. Aurich, U. Schonherr, H. Hoppe, C. Aurich, Effect of antioxidants on motility and membrane integrity of chilled-stored stallion semen, Theriogenology 48 (1997) 185–192.
[11] J.L. Bailey, J.F. Bilodeau, N. Cormier, Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon, J. Androl. 21 (2000) 1–7.
[12] J.F. Bilodeau, S. Chatterjee, M.A. Sirard, C. Gagnon, Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing, Mol. Reprod. Dev. 5 (2000) 282–288.
[13] D.E. Brooks, Carnitine, acetylcarnitine and the activity of carnitine acyltransferases in seminal plasma and spermatozoa of men, rams and rats, J. Reprod. Fert. 56 (1979) 667–773.
[14] M.N. Bucak, A. Atesşahin, A. Yuce, Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze–thawing process, Small Rumin. Res. 75 (2008) 128–134.
[15] M.N. Bucak, O. Uysal, The role of antioxidants in the freezing of Saanen goat semen, J. Indian Vet. 85 (2008) 148–150.
[16] M.N. Bucak, S. Sarıözkan, P.B. Tuncer, P.A. Ulutaş, H.A. Akcadağ, Effects of antioxidants on microscopic semen parameters, lipid peroxidation and antioxidant activities in Angora goat semen following cryopreservation, Rumin. Res. 81 (2009) 90–95.
[17] M.N. Bucak, P.B. Tuncer, S. Sarıözkan, N. Başpınar, M. Tasşpınar, K. Coyan, A. Bilgili, P.P. Akalın, S. Buyukleblebici, S. Aydos, S. Ilgaz, A. Sunguroğlu, D. Oztuna, Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage, Cryobiology 61 (2010) 248–253.
[18] M.N. Bucak, S. Sarıözkan, P.B. Tuncer, F. Sakin, A. Atessahin, R. Kulaksize, M. Çevik, The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities, Small Ruminant Res. 89 (2010) 24–30.
[19] M.N. Bucak, P.B. Tuncer, S. Sarıözkan, P.A. Ulutaş, K. Coyan, N. Başpınar, B. Ozkalp, Effects of hypotaurine, cysteamine and aminoacids solution on post-thaw microscopic and oxidative stress parameters of Angora goat semen, Res. Vet. Sci. 87 (2009) 468–472.
[20] M.N. Bucak, P.B. Tuncer, S. Sarıözkan, P.B. Ulutaş, Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters,

lipid peroxidation and anti-oxidant activities, Small Ruminant Res. 81 (2009) 13–17.
[21] W.M. Buckett, M.J.M. Luckas, I.A. Aird, R.G. Farquharson, C.R. Kingsland, D.I. Lewis-Jones, The hypo-osmotic swelling test in recurrent miscarriage, Fertil. Steril. 68 (1997) 506–509.
[22] P. Cassani, M.T. Beconi, C. O'Flaherty, Relationship between total superoxide dismutase activity and lipid peroxidation, dynamics and morphological parameters in canine semen, Anim. Reprod. Sci. 86 (2005) 163–173.
[23] E.T. Donnelly, N. McClure, S.E. Lewis, Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species, Mutagenesis 15 (2000) 61–68.
[24] W.C.L. Ford, J.M. Rees, The bioenergetics of mammalian sperm motility, in: C. Gagnon (Ed.), Controls of Sperm Motility: Biological and Clinical Aspects, CRC Press, Boca Raton, 1990, pp. 175–202.
[25] H. Funahashi, T. Sano, Selected, Sano Selected antioxidants improve the function of extended boar semen stored at 10 °C, Theriogenology 63 (2005) 1605–1616.
[26] L. Gandini, F. Lombardo, D. Paoli, L. Caponecchia, G. Familiari, C. Verlengia, F. Dondero, A.C. Lenzi, Study of apoptotic DNA fragmentation in human spermatozoa, Hum. Reprod. 15 (2000) 830–839.
[27] D.L. Garner, E.S.E. Hafez, Spermatozoa and seminal plasma, in: E.S.E. Hafez (Ed.), Reproduction in Farm Animals, Lea & Febier, Philadelphia, 1993, pp. 165–187.
[28] J.F. Griveau, E. Dumont, P. Renard, J.P. Callegari, D. Le Lannou, Reactive oxygen species, lipid peroxidation and enzymatic defense systems in human spermatozoa, J. Reprod. Fertil. 103 (1995) 17–26.
[29] G. Haines, B. Marples, P. Daniel, I. Morris, DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay, Adv. Exp. Med. Biol. 444 (1998) 789–791.
[30] P.R. Heaton, C.F. Reed, S.J. Mann, R. Ransley, J. Stevenson, C.J. Charlton, B.H. Smith, E.J. Harper, J.M. Rawlings, Role of dietary antioxidants to protect against DNA damage in adult dogs, J. Nutr. 132 (2002) 1720–1724.
[31] B.S. Jeong, Y. Xiangzhong, Cysteine, glutathione, and percoll treatments improve porcine oocyte maturation and fertilization in vitro, Mol. Reprod. Dev. 59 (2001) 330–335.
[32] M. Khlifaoui, I. Battut, J.F. Bruya, G. Chatagnon, A. Trimeche, D. Tainturier, Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism of action at spermatozoa level, Theriogenology 63 (2005) 138–149.
[33] L.M. Lewin, S. Fournier-Delpech, R. Weissenberg, R. Golan, T. Cooper, C. Pholpramool, Effects of pivalic acid and sodium pivalate on L-carnitine concentrations in the cauda epididymidis and on male fertility in the hamster, Reprod. Fertil. Dev. 9 (1997) 427–432.
[34] D. Mazor, E. Golan, V. Philip, Red blood cell permeability to thiol compounds following oxidative stress, J. Eur. Haematol. 57 (1996) 241–246.
[35] A. Michael, C. Alexopoulos, E. Pontiki, D. Hadjipavlou-Litina, P. Saratsis, C. Boscos, Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen–thawed canine spermatozoa, Theriogenology 68 (2007) 204–212.
[36] B. Novotna, J. Topinka, I. Solansky, I. Chvatalova, Z. Lnenickova, R.J. Sram, Impact of air pollution and genotype variability on DNA damage in Prague policemen, Toxicol. Lett. 172 (2007) 37–47.
[37] O. Ostling, K.J. Johanson, Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells, Biochem. Biophys. Res. Commun. 123 (1984) 291–298.
[38] R.C. Patra, D. Swarup, S.K. Dwivedi, Antioxidant effects of alpha-tocopherol, ascorbic acid and L-methionine on lead induced-oxidative stress to the liver, kidney and brain in rats, Toxicology 162 (2001) 81–88.
[39] K.T. Rajesh, K. Doreswamy, B. Shrilatha, M. Muralidhara, Oxidative stress associated with DNA damage in testis of mice: induction of abnormal sperms and effects on fertility, Mutat. Res. 513 (2002) 103–111.
[40] D.J. Reed, Glutathione: toxicological implications, Annu. Rev. Pharmacol. Toxicol. 30 (1990) 603–631.
[41] D.J. Reed, S. Orrenius, The role of methionine in glutathione biosynthesis by isolated hepatocytes, Biochem. Biophys. Res. Commun. 77 (1977) 1257–1264.
[42] S.G. Revell, R.A. Mrode, An osmotic resistance test for bovine semen, Anim. Reprod. Sci. 36 (1994) 77–86.
[43] E. Ruiz-Pesini, E. Alvarez, J.A. Enriquez, M.J. Lopez-Perez, Association between seminal plasma carnitine and sperm mitochondrial enzymatic activities, Int. J. Androl. 24 (2001) 335–340.
[44] J. Sagara, K. Miura, S. Bannai, Cysteine uptake and glutathione level in fetal brain cells in primary culture and in suspension, J. Neurochem. 61 (1993) 1667–1671.
[45] S. Sarıözkan, M.N. Bucak, P.B. Tuncer, P.A. Ulutas, A. Bilgen, The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation, Cryobiology 58 (2009) 134–138.
[46] S. Schafer, A. Holzmann, The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa, Anim. Reprod. Sci. 59 (2000) 201–211.
[47] N.P. Singh, R.E. Stephens, X-ray induced DNA double-strand breaks in human sperm, Mutagenesis 13 (1998) 75–79.
[48] B.T. Storey, Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa, Mol. Hum. Reprod. 3 (1997) 203–213.
[49] G. Stradaioli, L. Sylla, R. Zelli, P. Chiodi, M. Monac, Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions, Theriogenology 62 (2004) 761–777.
[50] B. Szczesniak-Fabianczyk, M. Bochenek, Z. Smorag, F. Ryszka, Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure, Reprod. Biol. 3 (2003) 81–87.
[51] P.B. Tuncer, M.N. Bucak, S. Büyükleblebici, S. Sarıözkan, D. Yeni, A. Eken, P.P. Akalın, H. Kinet, F. Avdatek, M. Gündoğan, The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen, Cryobiology 61 (2010) 303–307.
[52] J. Twigg, N. Fulton, E. Gomez, D.S. Irvine, R.J. Aitken, Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants, Hum. Reprod. 13 (1998) 1429–1436.
[53] O. Uysal, M.N. Bucak, Effect of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen, Acta. Vet. Brno. 76 (2007) 383–390.
[54] O. Uysal, M.N. Bucak, İ. Yavaş, Ö. Varışlı, Effect of various antioxidants on the quality of frozen-thawed bull semen, J. Anim. Vet. Adv. 6 (2007) 1362–1366.
[55] P.F. Watson, Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function, Reprod. Fertil. Dev. 7 (1995) 871–891.